

## Detecting co-cultivation induced chemical diversity via 2D NMR fingerprints



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**Rediscovery of already known compounds is a major issue in microbial natural product drug discovery. In recent years, progress has been made in developing more efficient analytical approaches that quickly identify known compounds in a sample to minimise rediscovery. In parallel, whole genome sequencing of microorganisms has revealed their immense potential to produce secondary metabolites, yet the majority of biosynthetic genes remain silent under common laboratory culturing conditions. Therefore, increased research has focused on optimising culturing methods to activate the silent biosynthetic gene clusters. Co-cultivation of different microbial strains can activate biosynthetic gene clusters that remain silent under standard laboratory fermentations involving mono-cultures, hence, the technique has great potential for natural product drug discovery. However, innovative methods are still needed to evaluate the success of any co-cultured fermentation end-product. Here, the application of HSQC-TOCSY NMR spectra and subsequent PCoA to identify changes in the metabolite diversity induced through co-cultivation is described.**

In nature, microorganisms occur in dynamic communities and thus co-cultivation allows for interspecific interactions that resemble

natural microbial ecosystems more closely and trigger the production of cryptic metabolites<sup>1</sup>. Numerous examples of successful co-cultivation induced natural products, most often polyketides and non-ribosomal peptides, have been published and reviewed in the literature<sup>2–7</sup>. Such examples highlight the potential of co-culturing for natural product drug discovery. Chemometric-profiling approaches have been used to assess the induction of microbial metabolites through co-cultures<sup>5</sup>. Application of liquid chromatography coupled to UV or MS detectors is often used, but these techniques depend on the presence of chromophores or the ability of the natural products to ionise. NMR fingerprinting techniques have also been reported, by which the crude extract of mono- and co-cultures were fractionated and <sup>1</sup>H NMR spectra of the pure cultures profiles were compared to those of the co-culture<sup>2</sup>. The strengths of NMR profiling are that it provides insight into structural components of the extract, it is highly reproducible and provides universal detection<sup>8</sup>. We recently reported a technique using 2D heteronuclear single quantum correlation - total correlation spectroscopy (HSQC-TOCSY) NMR profiles to prioritise microbial strains that have higher potential to produce drug-like natural products<sup>9</sup>. Hereby, the advantage of using 2D NMR profiles is that chemical resolution is improved by spreading the structural information over two-dimensions and consequently allows assessment of unfractionated crude extracts.

For co-cultivation, four strains all originating from different species, namely *Streptomyces* sp. (USC-16018), *Micromonospora* sp. (USC-16046), *Nocardia* sp. (USC-16096) and *Staphylococcus aureus* (ATCC 157293), were chosen in order to assess a broad diversity of microbial strains originating from different genera. Liquid fermentation was favoured over the solid agar cultures as mixed fermentations can more easily be upscaled, while on agar cultures the interactions between the two strains are generally highly localised and therefore compound isolation becomes more challenging as often low quantities of the compounds are produced<sup>5</sup>. Mono- and co-cultures were fermented in liquid ASW-A media<sup>10</sup> and after two weeks of incubation, the microbial cells were separated from the media and extracted with methanol (MeOH). The liquid media phases were partitioned with ethyl acetate (EtOAc) to extract the metabolites that were diffused into the media. The crude extracts of the mono- and co-cultures were profiled using 2D HSQC-TOCSY NMR experiments. The advantage of this approach is that metabolite changes such as *de novo* production, and up- or down-regulation of metabolites in crude extracts can readily be detected in the spectra without fractionation of the crude extract. NMR further provides insights about the structural classes in the extract. This is particularly valuable for untargeted analysis, where a chemical profile is first established to assess chemical diversity within a

sample and subsequent chemometric analysis can be used to identify uniqueness and similarities in the metabolome and reduce redundancy within the set of samples.

Visual inspection of the mono-culture profiles of the MeOH cell extracts readily depicted differences in microbial metabolomes at the genus level. *Micromonospora* sp. (USC-16046) displayed resonances of high intensity associated with peptides that were not detected in the other three mono-cultures. Resonances associated with polyketide fragments were observed in the *Nocardia* sp. (USC-16096) mono-culture as well as numerous peptide-associated NMR signals and diverse aromatic resonances. The *Staphylococcus* species (ATCC 157293) profile appeared chemically less diverse but indicated the presence of unique polyketide signals and *Streptomyces* sp. (USC-16018) had the least diverse mycelia extract (Figure 1). Comparison of the HSQC-TOCSY spectra of the MeOH cellular mono- and co-culture extracts showed clearly that the co-culture between *Micromonospora* sp. (USC-16046) and *S. aureus* (ATCC 157293) had triggered *de novo* secondary metabolite production. Numerous aromatic resonances not observed in the mono-cultures of the two strains as well as several peptide-associated peaks could be identified in the HSQC-TOCSY NMR profile (Figure 1). Additional polyketide fragment resonances were

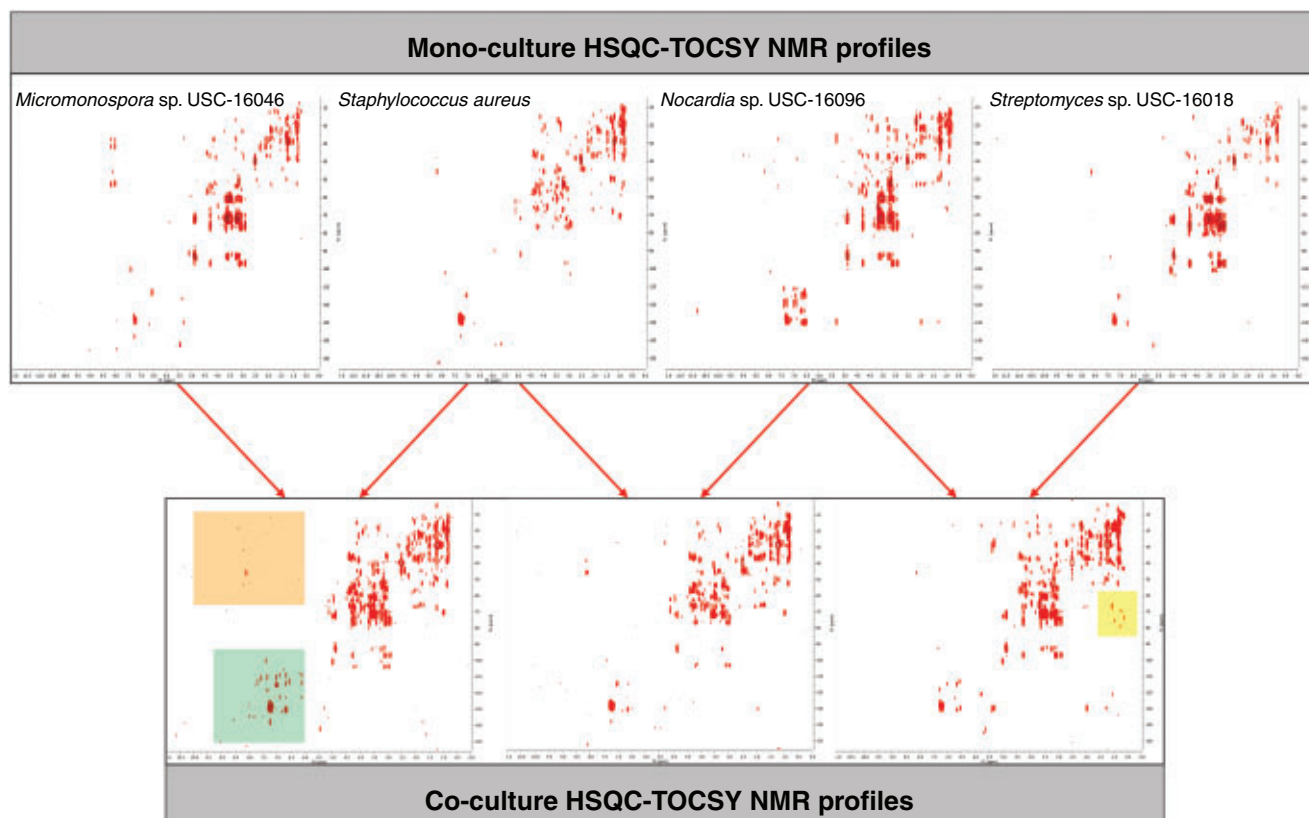


Figure 1. Example of HSQC-TOCSY NMR profiles of mono- and co-cultures of four microbial strains from four different genera showing induced polyketide resonances (yellow), peptide resonances (orange) and aromatic resonances (green).

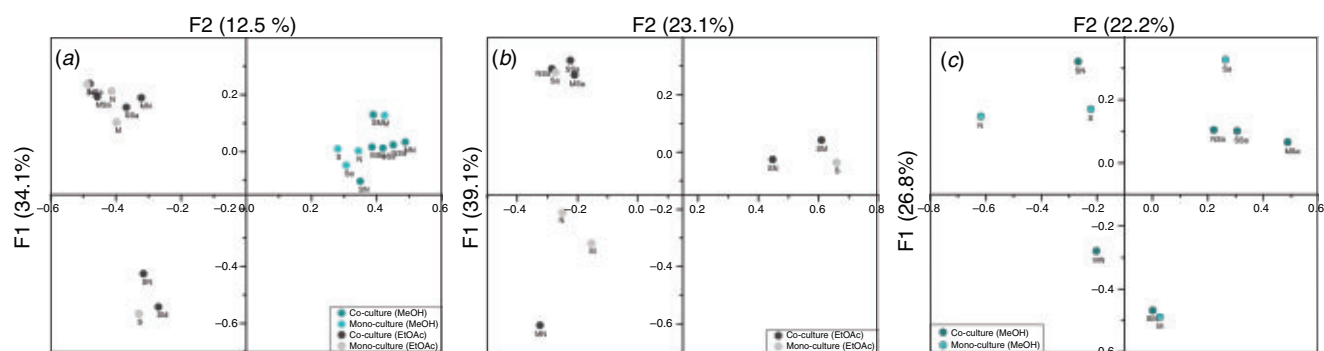


Figure 2. PCoA plot showing differences between (a) all microbial extracts (b) MeOH mycelia extracts and (c) EtOAc liquid culture media (S = *Streptomyces* sp., M = *Micromonospora* sp., N = *Nocardia* sp., Sa = *Staphylococcus aureus*).

observed in the co-culture of *Streptomyces* sp. (USC-16018) and *Nocardia* sp. (USC-16096) (Figure 1). The EtOAc extracts showed several resonances of compounds that had diffused into the culture media (NMR spectra not depicted).

A chemometric metabolomics approach using multivariate analysis was implemented in order to dereplicate the dataset and identify significant correlations and differences in the secondary metabolomes. Peaks were picked from the HSQC-TOCSY NMR spectra regions of interest that were associated with polyketide, peptide, double bond, aromatic and heteroaromatic structure fragments as previously described<sup>9</sup>. After removal of resonances found in the media control, peaks between the samples were aligned using an Excel macro. Dice similarities were calculated and the generated correlation matrix was used for Principle Coordinate Analysis (PCoA; Figure 2). The first two principal coordinates (F1/F2) were able to explain more than 45% of the variation within the dataset.

Based on the position of the mono-cultures compared to the co-culture it could be determined which of the two strains was more dominant in the culture (Figure 2). The co-culture of *Micromonospora* sp. (USC-16046) and *S. aureus* (ATCC 157293) was distinct to both mono-cultures reiterating the uniqueness of this co-culture previously observed in the HSQC-TOCSY profile.

The HSQC-TOCSY NMR profiling approach proved to be very useful for the assessment of co-cultures. Not only did the profiles provide insight about *de novo* induction of secondary metabolites but in some cases also their suppression. The *Micromonospora* sp. (USC-16046) peptide resonances, which were dominant in all other MeOH co-cultures with the species, were highly suppressed in the co-culture with *S. aureus* (ATCC 157293), while aromatic resonances were significantly enhanced. Furthermore, it was clearly evident that the microorganisms diffuse secondary metabolites into the media that significantly differ from the metabolites found in their mycelia. Therefore, it is valuable to consider a range of

extraction methods and solvents to obtain a complete spectrum of secondary metabolites in the culture.

Observations about the success and what type of structural fragments were found in the extracts could be made with minimal sample preparation in very little time as these 2D NMR spectra took only half an hour to be acquired. The inclusion of chemometric analysis can be a useful guide to detect unique samples, especially in larger datasets where sometimes similarities or differences in the NMR spectra cannot be identified solely by visual inspection. Only a few examples have been reported that utilise NMR profiles for the assessment of co-cultures<sup>2,11–13</sup>. Wu and co-workers implemented a <sup>1</sup>H-NMR-based metabolomics approach to study metabolomic changes in a *Streptomyces*-fungus co-culture and found significant differences between mono- and co-culture extracts, but only in combination with UHPLC-TOF-MS they were able to identify the compound class<sup>12</sup>. The here presented HSQC-TOCSY profiling approach is to our knowledge the first account where 2D NMR profiling was used on co-cultivations. Due to the increased chemical shift resolution through spreading of the detected signals across two planes in 2D NMR spectra, assessment of unfractionated crude extracts was permitted and immediately revealed the structure type of the induced secondary metabolites. In the future, it will be interesting to pursue large-scale fermentation of the co-culture between *S. aureus* (ATCC-157293) and either *Micromonospora* sp. (USC-16046) and or *Streptomyces* sp. (USC-16018) in order to identify the co-culture induced *de novo* produced secondary metabolites.

## Conflicts of interest

The authors declare no conflicts of interest.

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## Biographies

**Larissa Buedenbender** completed her doctoral studies in marine actinomycete biodiscovery at Griffith University, Australia, under the supervision of Anthony R. Carroll and D. İpek Kurtböke in 2018. Following her PhD, she has secured a post-doctoral position at the GEOMAR Helmholtz Centre for Ocean Research Kiel, Marine Natural Products Chemistry Research Unit in Kiel, Germany, where she is currently conducting marine natural products research.

**Anthony R Carroll** received his PhD from Sydney University and has been conducting research in marine natural products chemistry since his postdoctoral fellowships at the University of Hawaii and at James Cook University, Australia. Fifteen years as head of natural products chemistry for the AstraZeneca/Griffith University drug discovery project expanded his interests to include high throughput purification and structure determination techniques and cheminformatics. Since 2008 he has held a faculty position at Griffith University, Gold Coast where he is currently a Professor.

**D İpek Kurtböke** has been working in the field of biodiscovery and has been an active member of the international actinomycete research community since 1982. She currently conducts research and teaches in the field of applied microbiology and biotechnology and is senior lecturer at the University of the Sunshine Coast (USC), Queensland. She has also been an active member of the World Federation for Culture Collections (WFCC) including serving as the Vice-President of the Federation (2010–2013) and currently is the President of the Federation (2017–2020).

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